THE JOURNAL OF BIOLOGICAL CHEMISTRY
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Vol. 268, No. 2, Issue of January 15, pp. 1448-1455, 1993
Printed in U.S.A.

Expression and Structural Studies of Fasciclin I, an Insect Cell Adhesion Molecule*

(Received for publication, June 24, 1992, and in revised form, September 21, 1992)

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Fasciclin I is a lipid-linked cell-surface glycoprotein that can act as a homophilic adhesion molecule in tissue culture cells. It is thought to be involved in growth cone guidance in the embryonic insect nervous system. To facilitate structure-function studies, we have generated Chinese hamster ovary (CHO) cell lines expressing high levels of cell surface grasshopper and *Drosophila* fasciclin I. Grasshopper fasciclin I released by phospholipase C cleavage was purified on an immunoaffinity column and single crystals were obtained that diffracted to ~5-Å resolution. We also generated CHO and *Drosophila* S2 cell lines that produce a secreted form of fasciclin I. Fasciclin I expressed in S2 cells contains significantly less carbohydrate than the protein expressed in CHO cells, and may therefore be more suitable for crystallization. Biochemical characterization of purified fasciclin I indicates that the extracellular portion exists as a monomer in solution. Circular dichroism studies suggest that fasciclin I is primarily α-helical. Its structure is therefore different from other known cell adhesion molecules, which are predicted to be elongated β-sheet structures. This suggests that fasciclin I may define a new structural motif used to mediate adhesive interactions between cell surfaces.

Neural cell adhesion molecules determine many of the specific cell-cell interactions involved in the patterning of the nervous system. These include the pathway choices made by neuronal growth cones, bundling of neuronal processes, and synapse formation and rearrangement. Relatively little biochemical information concerning the molecular details of the recognition properties of adhesion molecules is available, primarily because they are membrane glycoproteins that are difficult to purify in large quantities. Although some of these molecules have been demonstrated to mediate homophilic adhesion when transfected into cells (1, 2), the possibility of heterophilic interactions with an unknown partner molecule is more difficult to test in a transfection assay. We report the development of an expression system to generate milligram amounts of a soluble form of an insect cell adhesion molecule, fasciclin I, as a first step in a biochemical and structural characterization of this neural adhesion molecule. This expression system could potentially be used to produce any adhesion molecule in a form that can be linked to a solid support and used to affinity-purify possible heterophilic ligand proteins.

Several novel cell adhesion molecules have been identified using monoclonal antibodies against neuronal membrane proteins from grasshopper and *Drosophila* embryos. The three fasciclins are expressed on subsets of central nervous system axons and are candidates for molecules involved in growth cone guidance during embryonic development (3). Another adhesion molecule, neuroglian, is more widely expressed on neurons and glia (3). Fasciclin II and neuroglian are members of the immunoglobulin (Ig)[1] superfamily and have domain organizations identical to those of the vertebrate neural cell adhesion molecules N-CAM and L1, respectively (4). Fasciclin III is distantly related to the Ig superfamily (3, 5). Fasciclin I contains four related domains, each of approximately 150 amino acids, that are not similar to other sequences in the current data bases (6). All four of the *Drosophila* molecules can function as homophilic adhesion molecules in transfected *Drosophila* Schneider 2 (S2) cells, and cell aggregates expressing different fasciclins sort away from each other (3, 5, 7).

In the embryo, *Drosophila* fasciclin I (72 kDa) and grasshopper fasciclin I (75 kDa) are expressed on the surfaces of all peripheral nervous system axons, a subset of central nervous system axons, and on some non-neuronal cells (6, 8, 9). Genetic evidence suggests that fasciclin I is involved in growth cone extension or guidance in the embryonic central nervous system. Flies bearing an apparent null mutation in the *fasciclin I* (*fasI*) gene are viable and do not exhibit visible defects in the central nervous system axon array. However, embryos doubly mutant in *fasI* and in *abl*, the *Drosophila* homolog of the Abelson tyrosine kinase proto-oncogene, have major defects in central nervous system axon pathways (10). This result suggests that fasciclin I and *abl* protein may be components of partially redundant signal transduction pathways involved in axon guidance.

We chose fasciclin I for our expression experiments because it has a novel primary sequence, and because it is linked to the cell surface by a glycosyl-phosphatidylinositol (GPI) linkage. This allows it to be released from the cell surface as a soluble molecule by treatment of intact cells with phosphatidylinositol-specific phospholipase C (PI-PLC). We show that

* This work was supported by the Howard Hughes Medical Institute (P. J. B.) and the National Institutes of Health (NS28182 to K. Z.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: Ig, immunoglobulin; CHO, Chinese hamster ovary; mAb, monoclonal antibody; PAGE, polyacrylamide gel electrophoresis; TBS, Tris-buffered saline; TBST, TBS-Tween; FPLC, fast protein liquid chromatography; αMEM, α minimum essential medium; DSP, 3,3′-dithio-*bis*-propionic acid *N*-hydroxysuccinimide ester; GPI, glycosyl-phosphatidylinositol; MSX, methionine sulfoximine; PI-PLC, phosphatidylinositol-specific phospholipase C.

grasshopper and *Drosophila* fasciclin I can be expressed in a GPI-linked form at high levels on the surface of mammalian cells, and in a secreted form when the sequence is truncated before the start of the GPI addition signal. Levels of protein expression were increased using a glutamine synthetase-based amplifiable expression system developed at Celltech, Berkshire, United Kingdom (11). When secreted from or expressed on the surface of Chinese hamster ovary (CHO) cells, the molecule contains an extra 15 kDa of carbohydrate not found on fasciclin I from insect embryos. Milligram quantities of soluble expressed fasciclin I have been purified on an immunoaffinity column, and large single crystals have been obtained. Perhaps because of the excess carbohydrate on the CHO-expressed protein, the crystals do not diffract to sufficient resolution to allow a structure determination by x-ray crystallographic methods. Preliminary studies in which the truncated form of grasshopper fasciclin I was transfected into *Drosophila* S2 cells show that the resulting secreted protein contains significantly less carbohydrate, which may make it more amenable to crystallization. Soluble fasciclin I expressed in CHO or S2 cells exists as a monomer in solution. This observation suggests that any homophilic interactions it mediates are of low affinity, or involve clusters of proteins on the cell surface. Circular dichroism analysis suggests that the structure of fasciclin I is primarily α-helical. This is in contrast to the predominantly β-sheet structures predicted for adhesion molecules that are members of the immunoglobulin superfamily (1, 2, 12) and/or contain fibronectin type III repeats (2).

MATERIALS AND METHODS

Reagents—Endoglycosidase F/*N*-glycosidase F was obtained from Boehringer Mannheim. The hybridoma cell line that produces 3B11, a mouse monoclonal antibody against grasshopper fasciclin I (8), and rat anti-grasshopper fasciclin I antiserum were gifts of Michael J. Bastiani (University of Utah). The monoclonal antibody 6D8 against *Drosophila* fasciclin I (13) was the gift of Corey Goodman (University of California, Berkeley). Goat anti-mouse fluorescein-conjugated IgG and goat anti-mouse IgG-peroxidase conjugate were obtained from Cappel Products. Mouse anti-rat IgG-alkaline phosphatase conjugate for Western blots was from Boehringer Mannheim. Methionine sulfoximine (MSX) and phospholipase C were from Sigma. The S2 cell line and the phsneo vector were gifts of Michael Jackson (Scripps Clinic). The pRmHa3 vector was the gift of Allan Bieber (Purdue University). All other chemicals were reagent-grade.

Construction of Lipid-linked and Soluble Forms of Grasshopper Fasciclin I—Molecular cloning experiments were performed by standard methods (14). Lipid-linked forms of fasciclin I were constructed by introducing full-length cDNA clones encoding grasshopper and *Drosophila* fasciclin I (6) into the *Eco*RI site of the polylinker of the expression vector pBJ5 (15), generating plasmids pBJ5.Gf1 and pBJ5.Df1, respectively. The cDNA clones used for the constructions encoded type IV grasshopper fasciclin I and type II *Drosophila* fasciclin I (9), respectively. This nomenclature refers to the presence or absence of micro-exon sequences between domains 2 and 3. Type II *Drosophila* fasciclin I contains the micro-exon sequence SFK, and type IV grasshopper fasciclin I contains the micro-exon sequence GF (9). The glutamine synthetase minigene from the Celltech vector pSVLGS.1 was excised using *Aat*II and *Bam*HI, the ends were filled in using T4 polymerase, and the resulting fragment was blunt-end cloned into the filled-in *Xho*I site of pBluescript KS(+) (Stratagene). A 5.5-kilobase *Xho*I-*Sal*I fragment containing the glutamine synthetase minigene was then cloned into the unique *Sal*I site of pBJ5.Df1 to generate pBJ5.GS.Df1 or into one of the two *Sal*I sites of pBJ5.Gf1 in a partial digest to obtain pBJ5.GS.Gf1.

To obtain a secreted form of grasshopper fasciclin I, a stop codon followed by a *Bam*HI site was inserted after residue 634 by site-directed mutagenesis (16). pBJ5.GS.sGf1 was generated by introducing an *Eco*RV-*Not*I fragment containing the modified cDNA into the *Xho*I (filled-in) and *Not*I sites of the polylinker of pBJ5.GS (17).

For expression in insect cells, the modified cDNA encoding the truncated form of grasshopper fasciclin I was subcloned into the *Eco*RI and *Bam*HI sites of the polylinker of the expression vector pRmHa3 (18) to generate pRmHa3.sGf1.

Cell Culture and Transfection—COS7 cells were grown in monolayer culture in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 units/ml penicillin/streptomycin (GIBCO or Irvine Scientific). CHO K1 cells were grown in α minimum essential medium (αMEM; Irvine Scientific) supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 units/ml penicillin/streptomycin. S2 cells were grown in Schneider's medium (Sigma) supplemented with 12.5% fetal bovine serum, 2 mM glutamine, and 100 units/ml penicillin/streptomycin.

For transient transfections, COS7 cells were transfected by the DEAE-dextran method (12 μg of DNA/60-mm 70% confluent plate) (19, 20). A 10% Me_2SO shock for 2 min following transfection with DEAE-dextran was employed to increase expression levels. Three days after transfection, the cells were harvested, washed with phosphate-buffered saline, and suspended in homogenization buffer (20 mM Tris, pH 7.5, 1 mM imidazole, 1 mM dithiothreitol, 1 mM $MgCl_2$, 20 mM sodium diphosphate, 1 mM phenylmethanesulfonyl fluoride, 1 μM pepstatin, 1 μM leupeptin). The cell extracts were then subjected to a chloramphenicol acetyltransferase assay (21) to determine the effectiveness of the transient transfection, or were prepared for Western blot analysis.

The pBJ5.GS.Gf1 and pBJ5.GS.sGf1 expression vectors were transfected into CHO cells by a liposome (Lipofectin)-mediated method (Bethesda Research Laboratories). Selection and amplification of the glutamine synthetase gene were carried out according to the protocol established by Celltech. In brief, 200 μl of DNA-Lipofectin complexes (30 μg of DNA plus 80–100 μg of Lipofectin) were introduced into a 10-cm plate with 50% confluent cells cultured in 1% dialyzed fetal bovine serum, αMEM without glutamine. After 18–24 h of incubation at 37 °C, the medium was replaced with 10% dialyzed fetal bovine serum, αMEM without glutamine and the incubation continued for an additional 24 h. The cells were then split into six 96-well plates and selected with 25 μM MSX in 10% dialyzed fetal bovine serum, αMEM without glutamine. MSX-resistant clones were isolated 2 weeks later. Positive transfectants were recloned and amplified under 100–500 μM MSX in 10% dialyzed fetal bovine serum, αMEM without glutamine. For cells transfected with pBJ5.GS.Gf1, the highest expressing cell populations were selected by immunofluorescence with 3B11 or flow cytometry of cells stained with 3B11. For the selection of clones expressing high levels of the secreted form of protein, cell supernatants were filtered through a nitrocellulose paper using a Minifold II slot blot system (Schleicher & Schuell model SRC072/0), and the presence of secreted fasciclin I was verified by immunostaining. The highest expressing cell lines were grown in a Cell Pharm II hollow-fiber bioreactor device (Unisyn Fibertec, San Diego, CA).

For stable transfection using the insect expression system, 25 μg of pRmHa3.sGf1 plus 2.5 μg of phsneo, a selection vector, were cotransfected into S2 cells (10^6 cells/ml) cultured in 10-cm plate (10 ml of medium) using a calcium phosphate procedure (Stratagene). Two days after transfection, cells were split into two 175-cm^2 flasks and selected with 1.0–1.6 mg/ml G418 (GIBCO). After 2 weeks, cells were passaged for a few generations and cloned in soft agar.[2] In brief, 8 ml of wild type S2 cells (10^5 cells/ml) and transformed cells (100–800 cells) in Schneider's medium, 12.5% fetal bovine serum, penicillin/streptomycin, with 1.0–1.6 mg/ml G418 were pipetted onto one side of a 10-cm bacteriological-grade Petri dish, leaving most of the surface of the plate uncovered. Two ml of a 1.5% molten agar solution (48 °C; Difco 0142-02) were pipetted onto the opposite side of the Petri plate. The contents of the plate were mixed thoroughly by swirling, and the plates were allowed to solidify and sealed with parafilm. Within 2 weeks of culture at 26 °C, clones became visible and were isolated and grown in individual wells of a 96-well plate. Expression of protein was induced for 2–3 days in serum-free medium containing 0.7 mM $CuSO_4$, and supernatants from individual clones were examined by Western blotting or using the Minifold II slot blot system.

Gel Electrophoresis and Western Blot Analysis—Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (22). Protein transfer after SDS-PAGE to nitrocellulose paper was done at 100 V for 3 h in transfer buffer (25 mM Tris, 190 mM glycine, 10% methanol) (23). Blots were immersed in 5% nonfat dry milk in TBST solution (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 30 min and then incubated

[2] A. Bieber, unpublished data.

with the primary mAb (6D8) at dilution of 1:1000 with TBST. After 1 h, the blots were washed for 10–15 min three times with TBST, twice with TBS (10 mM Tris-HCl, pH 8.0, 150 mM NaCl), and incubated with affinity-purified horseradish-peroxidase-conjugated goat anti-mouse IgG or mouse anti-rat (1:200, diluted with TBS, Cappel) for 30 min. After three washes with TBS, two with phosphate-buffered saline, and one with 50 mM Tris, pH 7.6, the blots were visualized using a diaminobenzidine-hydrogen peroxide staining solution (0.06% diaminobenzidine (w/v), 0.03% H_2O_2 (v/v) in 50 mM Tris, pH 7.6) until the bands were suitably dark (about 5 min). All procedures were carried out at room temperature.

Flow Cytometric Analysis—Trypsinized cells (2×10^6 cells/sample) were incubated with mAbs in ascites fluid at a dilution of 1:100 for at least 15 min on ice, washed three times with phosphate-buffered saline, and incubated with fluorescein-conjugated goat anti-mouse IgG. After 15 min, cells were washed three times with phosphate-buffered saline, resuspended in 1 ml of phosphate-buffered saline containing 0.02% sodium azide, and passed through a 50 μM filter. Flow cytometric analyses were carried out on Ortho Cytofluorograf 50H with a Data General 2150 computer system.

Enzymatic Characterization of Fasciclin I—Protein from transfected cells and from 10–14-h-old *Drosophila* embryos were deglycosylated with endoglycosidase F carried out according to the manufacturer's specifications (Boehringer Mannheim). Deglycosylated protein was analyzed on Western blots.

Purified grasshopper fasciclin I from CHO cells was treated with a battery of proteases, including trypsin, chymotrypsin, elastase, and papain. The protein was mixed with each protease at a 100:1 (w/w) ratio in phosphate-buffered saline (pH 7.1) and incubated at 37 °C. Aliquots were withdrawn at 0, 30, 60, and 120 min, the reaction stopped by adding phenylmethanesulfonyl fluoride to 2 mM, and samples run on a 8% SDS-PAGE gel.

For analytical assays, PI-PLC from *Bacillus cereus* (Boehringer Mannheim) was added to a final concentration of 1 unit/ml, and incubated for 1 h at 37 °C. For releasing fasciclin I from cells grown in the hollow fiber bioreactor device, phospholipase C containing PI-PLC (Sigma P6135; 1 mg/ml) was injected into the cell site at a 50-fold dilution for 3 h at 37 °C.

^{35}S Metabolic Labeling—10^5 transfected cells expressing GPI-linked grasshopper fasciclin I were cultured overnight with 100 μCi of [^{35}S] methionine. Cells were then washed and treated with PI-PLC. The resultant supernatants were concentrated 10-fold and were electrophoresed on a 10% SDS-PAGE gel. Gels were fixed and prepared for autoradiography using the EN^{3}HANCE™ system according to the manufacturer's directions (Du Pont).

N-terminal Amino Acid Sequencing—Cells from two 175-cm^2 flasks were washed thoroughly and treated with PI-PLC, and the supernatants were concentrated 30-fold using an Amicon Centricon-30 (M_r cutoff of 30,000). Concentrated supernatants were run on a 10% SDS-PAGE gel, electroblotted in a Bio-Rad transblot system onto a polyvinylidene difluoride membrane (Millipore), and inserted in an Applied Biosystems model 4778 sequenator reaction cartridge.

Purification of Soluble Grasshopper by Affinity Chromatography—The 3B11-protein A-coupled immunoaffinity column was prepared according to Harlow and Lane (24). Briefly, 5 ml of 3B11 ascites (2 mg/ml 3B11) were adjusted to pH 9.0, 3 M NaCl and then reacted with 3 ml of protein A beads (Pharmacia or Bio-Rad) for 1–2 h with gentle agitation. After coupling, beads were washed thoroughly with 50 mM sodium borate, 3 M NaCl, pH 9.0, four times and were resuspended in 10 volumes of 0.2 M sodium borate, 3 M NaCl, pH 9.0. Cross-linking of 3B11 and protein A beads was done using 20 mM dimethyl pimelimidate (Pierce Chemical Co.) in 0.2 M sodium borate, 3 M NaCl, pH 9.0 for 30 min. The reaction was stopped by washing once in 0.2 M ethanolamine (pH 8.0), and incubation was continued for additional 2 h. The beads were washed three times with phosphate-buffered saline and resuspended in phosphate-buffered saline with 0.04% sodium azide.

To purify grasshopper fasciclin I, supernatants from transfected CHO or *Drosophila* cells (about 500 ml) were passed over the affinity column at a flow rate of 20 ml/h at 4 °C, and the column was washed with 500 ml of phosphate-buffered saline, 0.05% sodium azide. Fasciclin I was then eluted with 0.1 M sodium acetate, pH 3.4. Fractions of 3 ml were collected in test tubes containing 0.5 ml of 1 M phosphate, pH 8.2, for immediate neutralization. Generally, about 1 mg of grasshopper fasciclin I was obtained per daily harvest from the Cell Pharm containing cells expressing the GPI or secreted form of grasshopper fasciclin I. About 0.5 mg of protein was obtained from 1 liter of S2 cells (5×10^6 cells/ml) after induction by 0.7 mM copper sulfate for 4 days.

Size-exclusion Chromatography—Following immunoaffinity chromatography, fasciclin I was concentrated to approximately 0.2–1 mg/ml using a vacuum dialysis apparatus (Schleicher & Schuell) and an Amicon Centricon-30 (M_r cutoff of 30,000). 50 μl of the concentrated protein were run on an FPLC Superose 12 column equilibrated with phosphate-buffered saline.

Cross-linking—Purified grasshopper fasciclin I (20 μl at 0.01–0.02 mg/ml) was treated for 30 min at 4 °C with 3,3′-dithio-*bis*-propionic acid *N*-hydroxysuccinimide ester (DSP; 40 mg/ml as a stock in dimethylformamide), a thiol-cleavable homobifunctional cross-linking reagent, at concentrations ranging from 100 to 800 μg/ml. Cross-linking reactions were stopped by addition of glycine to 50 mM, and samples were analyzed by Western blotting an SDS-PAGE gel run under reducing and nonreducing conditions.

Crystallization—Crystallization trials were conducted in hanging drops using the method of vapor diffusion (25). Hanging drops of grasshopper fasciclin I (4.2–4.6 mg/ml) in 10 mM Hepes, 0.02% NaN_3, pH 7.5, were prepared by mixing equal volumes of the protein and the well precipitant on a coverslip, which was then inverted and equilibrated over a well (24-well plate, Linbro) containing 1 ml of the undiluted precipitant. Crystals were grown in 1.84 M ammonium sulfate, pH 7.5–8.5, or 1.6 M Na,K phosphate, pH 7.5–8.5.

Circular Dichroism—The spectrum of CHO-derived secreted grasshopper fasciclin I (0.21 mg/ml in 5 mM sodium phosphate, pH 7.5) was collected using a Jasco J-600 spectropolarimeter. A cuvette with an 0.1-cm path length was used, and the mean residue ellipticity (degrees·cm^2/dmol) was determined assuming a mean residue weight of 141. All spectra were recorded at room temperature from 260 to 185 nm and were determined as the average of five scans.

RESULTS

Expression of GPI-linked and Soluble Forms of Fasciclin I in Mammalian and Insect Cell Lines—To express fasciclin I in mammalian cells, full-length cDNAs encoding the grasshopper and *Drosophila* fasciclin I proteins (6) were subcloned into the expression vector pBJ5 (15, 26), which contains a strong SV40/HTLV-1 hybrid promoter, as well as the SV40 origin of replication. The fasciclin I expression plasmids were transiently transfected into COS7 cells, in which plasmids bearing SV40 origins are efficiently replicated. After 3 days, cells transfected with the grasshopper fasciclin I plasmid were analyzed by immunofluorescent staining with the mAb 3B11, which recognizes grasshopper fasciclin I. 1–5% of the cells displayed bright surface staining (data not shown).

COS7 cells transfected with the *Drosophila* fasciclin I plasmid were treated with PI-PLC, and samples of supernatant and membranes were analyzed for the presence of fasciclin I by Western blotting with the mAb 6D8. Almost all of the fasciclin I appeared in the supernatant fraction, indicating that it could be released from the transfected cells by PI-PLC (data not shown). Because only a limited amount of antiserum capable of recognizing grasshopper fasciclin I on a Western blot was available, a comparable experiment was not performed on COS7 cells transfected with the grasshopper fasciclin I plasmid. However, similar data were obtained for grasshopper fasciclin I expressed in CHO cells (see below).

Having shown that fasciclin I could be expressed on the surface of mammalian cells, stable transformant lines were created in CHO cells. The glutamine synthetase minigene was first subcloned into pBJ5.Gf1 and pBJ5.Df1 to create the plasmids pBJ5.GS.Gf1 and pBJ5.GS.Df1. The glutamine synthetase gene can be used as a selectable marker and means of gene amplification in the presence of the drug MSX, a system developed at Celltech, Berkshire, United Kingdom (11). The glutamine synthetase-fasciclin I plasmids were introduced into CHO cells, and stable transformant lines were selected and amplified with increasing concentrations of MSX. Analysis of the highest expressing grasshopper fasciclin I line by fluorescence-activated cell sorting using the mAb 3B11

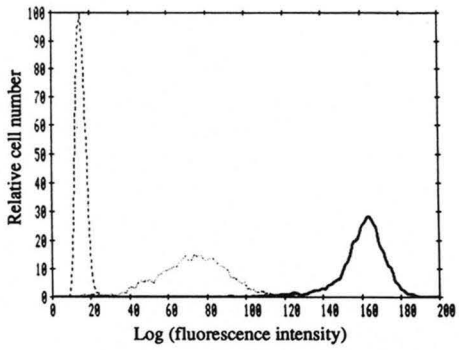

FIG. 1. **Flow cytometric analysis of the lipid-linked form of grasshopper fasciclin I expressed on CHO cells.** Staining of transfected cells by 3B11 mAb; secondary antibody is fluorescein-labeled goat anti-mouse IgG. ——, primary and secondary antibodies; – – –, secondary antibody alone; · · ·, primary and secondary antibody staining after treatment with PI-PLC.

showed that most of the fasciclin I could be released from these cells by PI-PLC (Fig. 1).

We were unable to analyze the *Drosophila* fasciclin I-expressing lines by fluorescence-activated cell sorting because none of the existing anti-fasciclin I mAbs, which had been raised against denatured protein expressed in *E. coli* (13), were capable of staining the surfaces of live cells. Since these mAbs could not be used for purification by affinity chromatography,[3] we did not attempt purification of *Drosophila* fasciclin I expressed in CHO cells. We have since used native protein released from the CHO-*Drosophila* fasciclin I line to generate new mAbs that stain live cells and can be used for affinity chromatography,[4] reagents that should be useful for future studies.

To characterize the CHO-derived grasshopper and *Drosophila* fasciclin I, cells were metabolically labeled with [^{35}S] methionine, and supernatatants from cells after PI-PLC treatment were analyzed on a 10% SDS-PAGE gel (Fig. 2*A*). The PI-PLC-released grasshopper and *Drosophila* fasciclin I migrated with apparent molecular masses of 90 and 85 kDa respectively, as compared to PI-PLC-released fasciclin I from insect embryos, which migrates with an apparent molecular mass of 75 kDa (grasshopper; Ref. 27) or 72 kDa (*Drosphila*; Ref. 13). A preparative amount of the 90-kDa band was isolated and subjected to N-terminal amino acid sequencing. The sequence obtained, KGEKSLEYKIRDDPDL, exactly corresponds to the N-terminal 16 residues of mature grasshopper fasciclin I (6, 27). The difference in molecular weight between CHO-derived grasshopper and *Drosophila* fasciclin I is probably due to the presence of six potential *N*-linked glycosylation sites in the grasshopper fasciclin I sequence, as compared to four in *Drosophila* fasciclin I (6).

Complex oligosaccharides are added to proteins synthesized in cells from vertebrate hosts, as compared to small oligosaccharide cores that are added to proteins synthesized in insect cells (28, 29). Thus, it seemed likely that the difference in molecular weight between the insect and CHO-derived fasciclin I proteins was due to additional glycosylation in CHO cells. To examine this possibility, PI-PLC-released protein from CHO lines was treated with endoglycosidase F and compared on a Western blot to similarly treated fasciclin I from *Drosophila* embryos. After deglycosylation, *Drosophila* fasciclin I from CHO cells comigrates with fasciclin I derived from embryos. Deglycosylated CHO-derived grasshopper fasciclin I also migrates at a similar position, corresponding to an apparent molecular mass of 68 kDa (Fig. 2*B*).

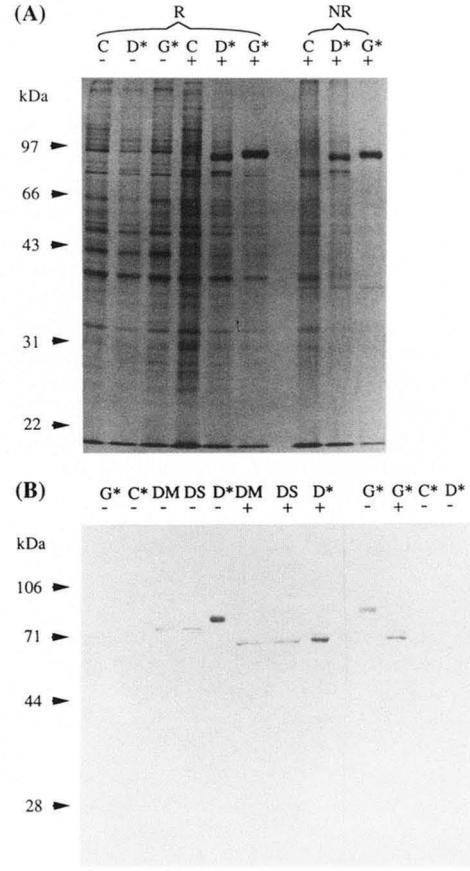

FIG. 2. **Characterization of grasshopper fasciclin I expressed in CHO cells by ^{35}S metabolic labeling (*A*) and deglycosylation with endoglycosidase F (*B*).** *A*, autoradiogram of 10% SDS-PAGE gel run under reducing (*R*) and nonreducing (*NR*) conditions. Samples are supernatants from 10^5 transformed cells that were cultured overnight with [^{35}S]methionine (100 μCi), and further incubated in the presence and absence of PI-PLC. *C*, untransfected CHO cells; *D**, CHO cell line expressing *Drosophila* fasciclin I; *G**, CHO cell line expressing grasshopper fasciclin I; –, no PI-PLC treatment; +, treated with PI-PLC. *B*, Western blots comparing molecular weights of deglycosylated CHO-derived *Drosophila* and grasshopper fasciclin I to fasciclin I derived from *Drosophila* embryos. Western blots of SDS-(8%) PAGE gels were probed with mAb 6D8 (*first eight lanes*) or a rat anti-grasshopper fasciclin I antiserum (*last four lanes*). *C*, untransfected CHO cells; *D**, PI-PLC-released protein from CHO cell line expressing *Drosophila* fasciclin I; *G**, PI-PLC-released protein from CHO cell line expressing grasshopper fasciclin I; *DM*, membrane fractions of *Drosophila* embryo; *DS*, supernatant of *Drosophila* embryo membrane preparations after digestion of PI-PLC; –, no endoglycosidase F treatment; +, treated with endoglycosidase F.

For generation of ordered crystals, a chemically homogeneous protein is the preferred starting material. In order to eliminate a potential source of heterogeneity in purified fasciclin I caused by the portion of the GPI tail remaining after PI-PLC digestion, a directly secreted form of the protein was also expressed in CHO cells. A stop codon was introduced by site directed mutagenesis (16) after amino acid 634 of grasshopper fasciclin I, which corresponds to the position presumably encoding the beginning of the GPI linkage site in the fasciclin I cDNA (13, 30), and the CHO expression vector was reconstructed. This plasmid, pBJ.GS.sGf1, was introduced into CHO cells and amplified lines selected as described above. The highest expressing lines were identified by slot blot analysis using the mAb 3B11 or by Western blotting of cell supernatants using the rat anti-fasciclin I antiserum.

[3] W.-C. Wang, unpublished results.
[4] T.-Y. Kung and W.-C. Wang, unpublished results.

The highest expressing CHO clones making the GPI-linked and the secreted versions of grasshopper fasciclin I were grown in a Cell Pharm II hollow fiber bioreactor device (Unisyn Fibertec, San Diego, CA) in the presence of 100 or 300 μM MSX. The expressed protein was isolated from the medium by passing it over a 3B11 mAb immunoaffinity column after a 3-h phospholipase C incubation in the case of the GPI-linked fasciclin I, or with no incubation in the case of the truncated fasciclin I. Approximately 0.7–1.0 mg of pure protein per daily harvest from lines expressing either the GPI-linked or the secreted form of fasciclin I were obtained. Purified GPI-linked fasciclin I migrates with an apparent molecular mass of ~90 kDa (Fig. 3*A*) as compared to ~88 kDa for the secreted form (Fig. 3*B*) and is >90% pure as judged by a Coomassie stained SDS-PAGE gel (Fig. 3*A*).

In an effort to express high levels of fasciclin I containing the physiological amount of carbohydrate for biochemical analysis, the cDNA encoding the secreted form of grasshopper fasciclin I was also introduced into a vector containing a metallothionein promoter that functions at high levels in copper-induced *Drosophila* Schneider cell lines (18). The fasciclin I expression plasmid, pRmHa3.sGf1, was stably introduced into Schneider 2 (S2) cells by cotransfection with a selection vector, phsneo (31). The highest-expressing clones were identified by Western blotting of cell supernatants, and secreted fasciclin I was purified as described above. About 0.5 mg of fasciclin I/liter can be obtained from supernatants of these cells after copper induction, and this protein migrates with the expected molecular mass of 75 kDa on SDS-PAGE gels (Fig. 3*B*).

Biochemical Characterization of Purified Fasciclin I—Stable fragments corresponding to domain structures can often be generated by proteolytic treatment of proteins containing multiple domains. To examine the stability and possible domain structure of purified fasciclin I, samples of purified protein were treated with trypsin, chymotrypsin, papain, and elastase. Fasciclin I was resistant to digestion by all of these proteases (data not shown), suggesting that the protein is folded into a stable structure without obvious flexible linker sequences between its domains.

Drosophila fasciclin I has been shown to act as a homophilic adhesion molecule in tissue culture cells (7), so it was of interest to determine whether this adhesion activity would correlate with oligomerization in solution. The oligomeric structure of purified grasshopper fasciclin I was examined by gel filtration and cross-linking studies. Concentrated fasciclin I from CHO or S2 cells migrates on a Superose 12 FPLC column in a position corresponding to a monomeric rather than a dimeric or higher oligomeric structure (Fig. 4*A*). In addition, cross-linking of the S2-derived protein with the homobifunctional reagent DSP did not result in the appearance of dimeric or other oligomeric species on SDS-PAGE gels (Fig. 4*B*). Thus, our data suggest that the extracellular portion of fasciclin I exists as a monomer in solution. Similar results have been observed for the extracellular portions of the homophilic adhesion molecules N-CAM (32) and cadherins (32, 33).

Crystallization and Structural Characterization of Fasciclin I—Grasshopper fasciclin I purified after PI-PLC cleavage crystallizes in the orthorhombic space group $C222_1$ with unit cell dimensions 156 Å × 352 Å × 168 Å. Based on average volume to mass ratios (34), the asymmetric unit of the crystal is estimated to contain between 2 and 8 molecules (corresponding to solvent contents between 81% and 23%). Typical crystals grow to a size of 0.5 mm × 0.3 mm × 0.3 mm (Fig. 5). Using nickel-filtered CuKα radiation from a rotating anode

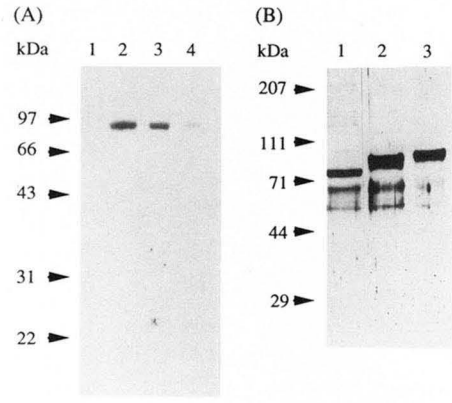

FIG. 3. *A*, SDS-(10%) PAGE gel of four fractions from affinity column purification of lipid-linked grasshopper fasciclin I expressed in CHO cells. Fasciclin I was eluted from the affinity column with a low pH buffer and four 3.0-ml fractions were collected. 10 μl from each fraction are shown on the gel. *B*, molecular weight comparison of three different forms of expressed grasshopper fasciclin I on Western blot of SDS-(10%) PAGE gel probed with a rat anti-grasshopper fasciclin I antiserum. *Lane 1*, secreted form expressed in S2 cells; *lane 2*, secreted form expressed in CHO cells; *lane 3*, GPI-linked form expressed in CHO cells after treatment with PI-PLC.

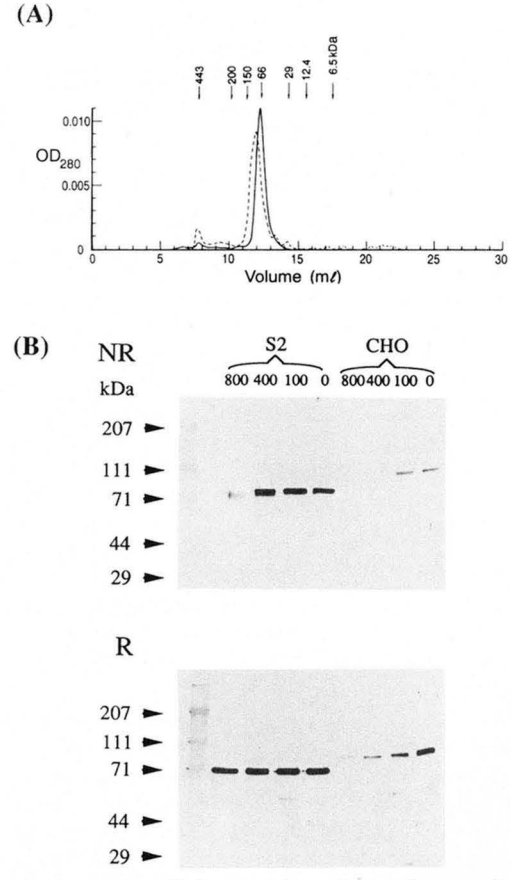

FIG. 4. **The extracellular portion of grasshopper fasciclin I exists as a monomer in solution, as demonstrated by gel filtration chromatography (*A*) and cross-linking (*B*).** *A*, gel filtration profile (FPLC Superose 12 column in a phosphate-buffered saline solution, flow rate 0.4 ml/min) of purified grasshopper fasciclin I secreted from CHO cells (– – –) or from *Drosophila* S2 cells (——). *B*, SDS-(8%) PAGE gels run under reducing (*R*) and nonreducing conditions (*NR*) of CHO- or S2-derived fasciclin I cross-linked treated with a reducible homobifunctional cross-linking reagent, DSP, at different concentrations of cross-linking reagents indicated by numbers above the lanes (in μg/ml).

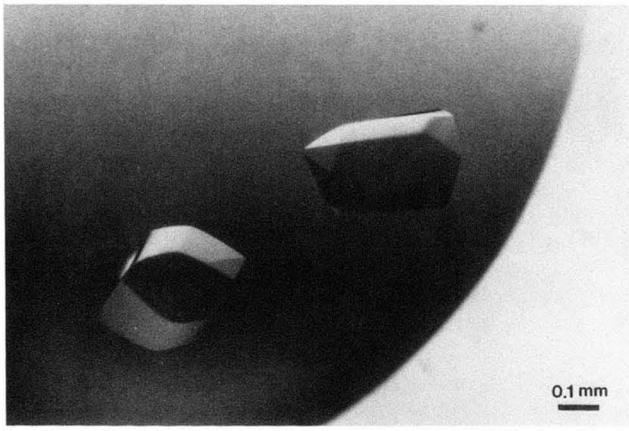

FIG. 5. **Crystals of CHO-derived grasshopper fasciclin I.** Length of crystals is indicated by the *bar*.

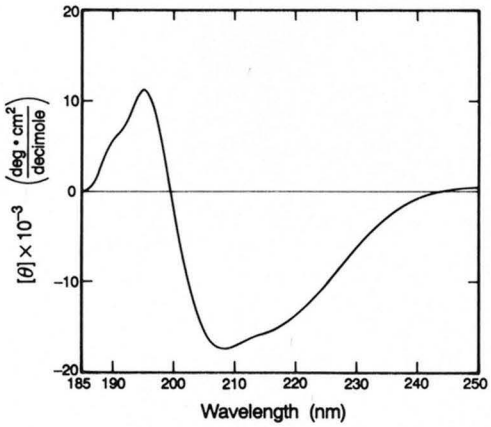

FIG. 6. **Far-UV CD spectrum of the secreted form of CHO-derived grasshopper fasciclin I (0.21 mg/ml in 5 mM sodium phosphate, pH 7.0) expressed as ellipticity per mean residue.**

x-ray generator, the crystals diffract to ~5 Å with anisotropic diffraction in one direction to 3.5 Å. Crystals of secreted grasshopper fasciclin I expressed in CHO cells are isomorphous and do not diffract to higher resolution. We did not obtain sufficient amount of secreted fasciclin I from the S2 expression system for crystallization trials.

To obtain information regarding the secondary structure content of fasciclin I, the far-UV CD spectrum of fasciclin I was analyzed. As shown in Fig. 6, the CD spectrum of fasciclin I is characterized by a positive peak at 195 nm followed by deep negative doublets at 208 nm and 220 nm, indicating a strong α-helical component. The ellipticity of a protein at any given wavelength in the far-UV region results from the sum of the contributions from the secondary structural elements within the molecule, thus allowing the estimation of the amounts of α-helix, β-sheet, β-turn, and irregular structure from the CD spectrum in this region (35). Based on an equation developed by Greenfield and Fasman (1969), the α-helical content of fasciclin I is calculated as 43% (36). A similar estimate of the percentage helical structure is obtained using the algorithm of Chang (37), in which analysis of the fasciclin I CD spectrum predicts 37% α-helix, 0% β-sheet, 19% β-turn, and 34% irregular structure. These results indicate that fasciclin I, unlike the adhesion molecules that are members of the immunoglobulin gene superfamily (1, 2) or contain fibronectin type III repeats (2, 38), is likely to contain a significant portion of α-helical structure.

DISCUSSION

Although grasshopper and *Drosophila* fasciclin I are closely related (6), only *Drosophila* fasciclin I has been demonstrated to mediate homophilic adhesion in tissue culture cells (7), and it is unclear whether homophilic adhesion is the primary function of fasciclins *in vivo*. After our experiments were initiated, it was found that S2 cells expressing grasshopper fasciclin I do not aggregate convincingly.[3,5] Thus, the functionality of purified grasshopper fasciclin I cannot be assayed by inhibition of cell aggregation. Two possible explanations for the failure of grasshopper fasciclin I expressing cell lines to aggregate are as follows: (i) other components in the S2 cells are required for aggregation, and these do not interact effectively with the heterologous grasshopper fasciclin I protein (grasshopper and *Drosophila* fasciclin I are only 48% identical in sequence; Ref. 6). The recent observation that cytoskeletal catenin molecules are necessary for aggregation of cadherin-expressing cells is an example of a requirement for a protein in addition to a cell surface adhesion molecule for demonstration of adhesion (39). (ii) Both *Drosophila* and grasshopper fasciclin I exist in three different forms. These forms differ by the inclusion of 2, 3, or 6 amino acids encoded by micro-exons between the second and third domains (9). *Drosophila* fasciclin I has either no micro-exon sequence (+0 form), the first micro-exon sequence (SFK; +3 form), or both micro-exon sequences (SFKFMN; +6 form). Grasshopper fasciclin I exists as the +0 and +3 (SFK) forms and an additional form containing only a second micro-exon sequence (GF; +2 form). No +6 form exists for grasshopper fasciclin I. Seeger and Goodman[5] have shown that *Drosophila* +0 and +3 fasciclin I mediate homophilic adhesion in S2 cells, but +6 does not, or does so much more weakly. The +2 form of grasshopper fasciclin I, which was the form originally cloned (6) and the one that has been used for all expression and aggregation experiments, may be equivalent to *Drosophila* +6. Thus, this form of fasciclin I may have a function other than, or in addition to, homophilic adhesion. To look for other activities of the purified fasciclin I, we added it to grasshopper embryo cultures in an attempt to perturb the formation of axon tracts. However, no obvious effects were observed.[6]

Despite the lack of a functional assay for grasshopper fasciclin I protein, we are confident that the molecule we have purified is correctly folded. Five lines of evidence support this conclusion. (i) Purified fasciclin I is resistant to digestion by several different proteases (see "Results"), the behavior of a compact globular protein rather than an aberrantly folded or denatured protein. (ii) The purified protein migrates as a sharp peak corresponding to a monomer on a gel filtration column (Fig. 4*A*), while partially folded or denatured proteins migrate as broad peaks or elute in the void volume due to aggregation. (iii) The CD analysis shows a spectrum characteristic of α-helical structure, not of a random coil (Fig. 6), and the spectrum converts to random coil in the presence of guanidine hydrochloride (data not shown). (iv) We have obtained crystals of fasciclin I. Crystallization of a protein requires an array of identically shaped molecules, whereas unfolded or aberrantly folded molecules (which exist as a mixture of species) cannot be crystallized. (v) The 3B11 mAb used to purify the protein, which does not recognize denatured fasciclin I (8),[3] can immunoprecipitate purified fasciclin I after elution from a 3B11 column, and purified fasciclin I rebinds to this column (data not shown).

Results from gel filtration chromatography and cross-linking studies suggest that the extracellular portion of grasshop-

[5] M. Seeger and C. S. Goodman, personal communication.
[6] B. Condron, M. Bastiani, and W.-C. Wang, unpublished results.

per fasciclin I is a monomer in solution. Thus, we do not observe an oligomerization activity in solution that correlates with the homophilic adhesion activity of *Drosophila* fasciclin I expressed on the surface of S2 cells (7). Similarly, formation of dimers or polymers of isolated extracellular domains of other homophilic adhesion molecules such as the cadherins (32, 33), or N-CAM (32) have never been observed. These results suggest that the homophilic interactions mediated by these adhesion molecules are of low affinity, and/or involve clusters of proteins on the cell surface. In the case of grasshopper fasciclin I, the lack of oligomerization is consistent with the lack of homophilic adhesion activity displayed by grasshopper fasciclin I in S2 cells.[3,5]

Crystals of purified fasciclin I do not diffract to a resolution sufficient for the determination of an atomic resolution structure. This may be due to the large amount of excess carbohydrate on the CHO-derived protein as compared to the amount of carbohydrate on fasciclin I expressed in insect cells. In an attempt to obtain fasciclin I with the physiological amount of carbohydrate, we expressed a secreted version of grasshopper fasciclin I in *Drosophila* S2 cells. However, because of low expression of fasciclin I in S2 lines, we have been unable to obtain sufficient protein bearing the normal amount of carbohydrate for extensive crystallization trials.

In the absence of high resolution structure information, an analysis of its circular dichroism spectrum was performed to allow us to compare the secondary structure composition of fasciclin I to that of other adhesion molecules. A number of adhesion molecules mediating interactions in the nervous and the immune systems consist of a series of repeated β-sheet domain structures. For example, the extracellular domains of N-CAM and fasciclin II are composed of five immunoglobulin C2-like domains followed by two fibronectin type III repeats (3, 40). ICAM-1, which interacts with the integrin LFA-1 on T cells, contains five immunoglobulin variable-like domains in its extracellular portion (41). The immunoglobulin variable domain fold has been well-characterized as a two β-sheet structure linked by a disulfide bond, and the crystal structure of a fragment of CD4 (42, 43) illustrates its use by an adhesion molecule that is a member of the immunoglobulin superfamily. Fibronectin type III repeats are also β-sheet structures, as predicted (44) and verified by the recent crystal structure of the human growth hormone receptor (38). A combination of electron microscopic studies (32, 41) with crystallographic studies of CD4 (42, 43, 45) suggest that adhesion molecules that are immunoglobulin superfamily members are likely to have extended structures with flexible linker regions between domains.

The primary sequence of fasciclin I suggests that it consists of four related domains arranged in tandem on a single polypeptide chain, similar to the domain organization of CD4 (46), but the fasciclin I repeats do not have homology to immunoglobulin domains or to any other protein in the current data bases (6). An analysis of the circular dichroism spectrum of purified grasshopper fasciclin I suggests that it is a predominantly α-helical protein, with little or no contribution from β-structure. Based upon the 48% sequence identity between *Drosophila* and grasshopper fasciclin I (6), the α-helical structure demonstrated for the grasshopper protein is expected to be shared by the *Drosophila* homolog as well (47). These results suggest that the structure of fasciclin I is substantially different than the structures of other invertebrate and vertebrate adhesion molecules, and that it may mediate adhesion through use of a novel structural motif. A human protein related to fasciclin I has been found,[7] demonstrating the existence of a vertebrate molecule using this structural motif. So far, a function in mediating homophilic adhesion has been demonstrated for two of the three forms of *Drosophila* fasciclin I, but the functions of the other forms of insect fasciclin I and of the human fasciclin I-related protein are unknown. It is likely that additional molecules will be found in both vertebrate and invertebrate systems that use a helical motif to mediate adhesive interactions, and it will be of interest to define their mode of interaction and function. The experiments described here are the initial steps toward a molecular characterization of this new family of proteins.

Acknowledgments—We thank Ilana Tamir and Jim Moore for help with CD analysis, Pantelis Tsoulfas for help with constructing the secreted form of fasciclin I, David Penny and Louis Gastinel for help with tissue culture, Chris Bebbington for the glutamine synthetase vector, and Peggy Fahnestock, Louis Gastinel, and Malini Raghavan for critically reading the manuscript.

REFERENCES

1. Jessell, T. M. (1988) *Neuron* **1,** 3–13
2. Hynes, R. O., and Lander, A. D. (1992) *Cell* **68,** 303–322
3. Grenningloh, G., Bieber, A. J., Rehm, E. J., Snow, P. M., Traquina, Z. R., Hortsch, M., Patel, N. H., and Goodman, C. S. (1990) *Cold Spring Harbor Symp. Quant. Biol.* **55,** 327–340
4. Harrelson, A. L., and Goodman, C. S. (1988) *Science* **242,** 700–708
5. Snow, P. M., Bieber, A. J., and Goodman, C. S. (1989) *Cell* **59,** 313–323
6. Zinn, K., McAllister, L., and Goodman, C. S. (1988) *Cell* **53,** 577–587
7. Elkins, T., Hortsch, M., Bieber, A. J., Snow, P. M., and Goodman, C. S. (1990) *J. Cell Biol.* **110,** 1825–1832
8. Bastiani, M. J., Harrelson, A. L., Snow, P. M., and Goodman, C. S. (1987) *Cell* **48,** 745–755
9. McAllister, L., Rehm, E. J., Goodman, C. S., and Zinn, K. (1992) *J. Neurosci.* **12,** 895–905
10. Elkins, T., Zinn, K., McAllister, L., Hoffmann, F. M., and Goodman, C. S. (1990) *Cell* **60,** 565–575
11. Bebbington, C. R., and Hentschel, C. C. G. (1987) *DNA Cloning: A Practical Approach* (Glover, D. M., ed) Vol. III, pp. 163–188, IRL Press, Oxford
12. Williams, A. F., and Barclay, A. N. (1988) *Annu. Rev. Immunol.* **6,** 381–405
13. Hortsch, M., and Goodman, C. S. (1990) *J. Biol. Chem.* **265,** 15104–15109
14. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
15. Lin, A. Y., Devaux, B., Green, A., Sagerström, C., Elliott, J. F., and Davis, M. M. (1990) *Science* **249,** 677–679
16. Kunkel, T. A., Roberts, J. D., and Zakour, R. A. (1987) *Methods Enzymol.* **154,** 367–382
17. Gastinel, L. N., Simister, N. E., and Bjorkman, P. J. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89,** 638–642
18. Bunch, T. A., Grinblat, Y., and Goldstein, L. S. B. (1988) *Nucleic Acids Res.* **16,** 1043–1061
19. Gorman, C. (1985) *DNA Cloning: A Practical Approach* (Glover, D. M., ed) Vol. II, pp. 143–190, IRL Press, Oxford
20. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1989) *Current Protocols in Molecular Biology,* Vol. I, Chap. 9, Wiley Interscience, New York, NY
21. Gorman, C., Moffat, L. F., and Howard, B. H. (1982) *Mol. Cell. Biol.* **2,** 1044–1051
22. Laemmli, U. K. (1970) *Nature* **227,** 680–685
23. Towbin, H., Staehelin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
24. Harlow, E., and Lane, D. (1988) *Antibodies: A Laboratory Manual,* pp. 522–523, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
25. Wlodawer, A. F., and Hodgson, K. O. (1975) *Proc. Natl. Acad. Sci. U. S. A.* **72,** 398–399
26. Takebe, Y., Seike, M., Fujisawa, J.-I., Hoy, P., Yokota, K., Arai, K.-I., Yoshida, M., and Arai, N. (1988) *Mol. Cell. Biol.* **8,** 466–472
27. Snow, P. M., Zinn, K., Harrelson, A. L., McAllister, L., Schilling, J., Bastiani, M. J., Makk, G., and Goodman, C. S. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 5291–5295
28. Carr, S. A., Hemling, M. E., Folena-Wasserman, G., Sweet, R. W., Anumula, K., Barr, J. R., Huddleston, M. J., and Taylor, P. (1989) *J. Biol. Chem.* **264,** 21286–21295
29. Kuroda, K., Geyer, H., Geyer, R., Doerfler, W., and Klenk, H.-D. (1990) *Virol.* **174,** 418–429
30. Ferguson, M. A. J., and Williams, A. F. (1988) *Annu. Rev. Biochem.* **57,** 285–320
31. Rio, D. C., and Rubin, G. M. (1985) *Mol. Cell. Biol.* **5,** 1833–1838
32. Becker, J. W., Erickson, H. P., Hoffman, S., Cunningham, B. A., and Edelman, G. M. (1989) *Proc. Natl. Acad. Sci. U. S. A.* **86,** 1088–1092
33. Takeichi, M. (1990) *Annu. Rev. Biochem.* **59,** 237–252
34. Matthews, B. W. (1968) *J. Mol. Biol.* **33,** 491–497
35. Greenfield, N., and Fasman, G. D. (1969) *Biochemistry* **8,** 4108–4116
36. Wang, W.-C. (1992) *Expression, Structural and Functional Studies of Fas-*

[7] S. Takeshita, R. Kikuno, K.-I. Tezuka, and E. Amann, personal communication.

ciclin I, an Insect Cell Adhesion Molecule. Ph.D. thesis, California Institute of Technology
37. Chang, T. C., Wu, C.-S. C., and Yang, J. T. (1978) *Anal. Biochem.* **91,** 13–31
38. de Vos, A. M., Ultsch, M., and Kossiakoff, A. A. (1992) *Science* **255,** 306–312
39. Hirano, S., Kimoto, N., Shimoyama, Y., Hirohashi, S., and Takeichi, M. (1992) *Cell* **70,** 293–301
40. Cunningham, B. A., Hemperly, J. J., Murray, B. A., Prediger, E. A., Brackenbury, R., and Edelman, G. M. (1987) *Science* **236,** 799–806
41. Staunton, D. E., Dustin, M. L., Erickson, H. P., and Springer, T. A. (1990) *Cell* **61,** 243–254
42. Wang, J., Yan, Y., Garrett, T. P. J., Liu, J., Rodgers, D. W., Garlick, R. L., Tarr, G. E., Husain, Y., Reinherz, E. L., and Harrison, S. C. (1990) *Nature* **348,** 411–419
43. Ryu, S.-E., Kwong, P. D., Truneh, A., Porter, T. G., Arthos, J., Rosenberg, M., Dai, X., Xuong, N.-h., Axel, R., Sweet, R. W., and Hendrickson, W. A. (1990) *Nature* **348,** 419–426
44. Bazan, J. F. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 6934–6938
45. Kwong, P. D., Ryu, S. E., Hendrickson, W. A., and Axel, R. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87,** 6423–6427
46. Maddon, P. J., Littman, D. R., Godfrey, M., Maddon, D., Chess, L., and Axel, R. (1985) *Cell* **42,** 93–104
47. Chothia, C. and Lesk, A. M. (1986) *EMBO J.* **5,** 823–826